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(71) Applicant *(for all designated States except US)*: RUTGERS, THE STATE UNIVERSITY OF NEW JERSEY [US/US]; Old Queens Building, Somerset and George Streets, New Brunswick, NJ 08903 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: DESAI, Shyamal, D. [US/US]; 190D Cedar Lane, Highland Park, NJ 08904 (US). LIU, Leroy, Fong [US/US]; 5 Fairacres Drive, Bridgewater, NJ 08807 (US). LAVOIE, Edmond, J. [US/US]; 3 Guilford Court, Princeton Junction, NJ 08550 (US).

(74) Agent: VIKSNINS, Ann, S.; Schwegman, Lundberg, Woessner & Kluth, P.O. Box 2938, Minneapolis, MN 55402 (US).

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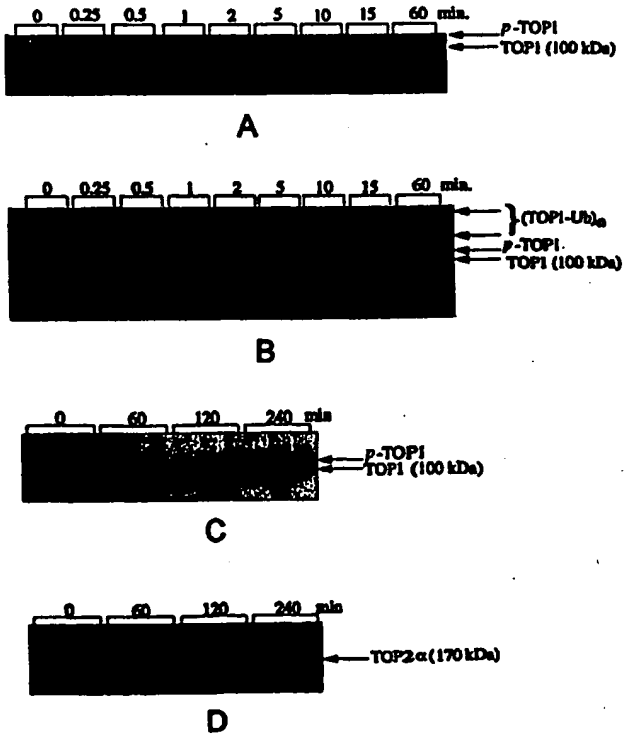
(57) Abstract

The present invention relates to methods for identifying cells with increased sensitivity to treatment with cytotoxic agents. The invention also relates to method for identifying cells with abnormal proteolytic processing mechanisms. More specifically, the invention relates to a method of using ubiquitin cross-reactive protein as a prognostic marker for tumor cells with enhanced sensitivity to treatment with DNA-damaging agents, including camptothecin and its analogs.



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UBIQUITIN CROSS-REACTIVE PROTEIN AS A PROGNOSTIC MARKER FOR TUMOR CELL CHEMOSENSITIVITY

Related Application

The invention described herein claims priority to U.S. Provisional Application Ser. No. 60/129,063, filed 13 April 1999, and U.S. Provisional Application Ser. No. 60/157,745, filed 05 October 1999, under 35 U.S.C. 119.

Statement of Government Rights

The invention described herein was made with government support under Grant Number CA39662, CA72878 and CA59750 awarded by the National Institutes of Health. The United States Government has certain rights in the invention.

Background of the Invention

Although a variety of approaches to cancer therapy, including surgical resection, radiation therapy, and chemotherapy, have been available and commonly used for a number of years, cancer remains one of the leading causes of death in the United States and the world. Many such treatments are described in CANCER: Principles & Practice of Oncology, 2nd Edition (1985), DeVita *et al.*, eds., J.B. Lippincott Company, Philadelphia, Pa. Since many forms of cancer exhibit rapid progression and are not readily detectable until there is widespread dissemination of the disease, it is critically important to the survival of the patient that the disease be detected as early as possible and that the most effective treatment be found at an early stage.

A number of chemotherapeutic agents have been developed for the treatment of various forms of cancer, most of which rely upon the most characteristic feature of tumor cells--rapid and relatively uncontrolled cell division. Among these agents is a large class of agents known to act as DNA-damaging agents, including alkylating agents such as melphalan, bisulfan and nitrosoureas, antimetabolites such as mercaptopurine, fluorouracil and methotrexate, topoisomerase I "poisons" such as camptothecin, bi- and terbenzimidazoles, as well as certain benzo[c]phenanthridine and protoberberine

alkaloids, and topoisomerase II poisons such as adriamycin, etoposide (VP-16), actinomycin D and daunomycin.

In clinical use, these agents have shown promise in the treatment of various forms of cancer. The challenge has become the identification of the appropriate agent for the treatment of a specific tumor type. Treatment with an ineffective chemotherapeutic agent costs the patient valuable time that could have been used for effective treatment, and during that time the disease often progresses. Furthermore, the progress of the disease may actually be aided by ineffective treatment, since many of the chemotherapeutic agents also depress the immune system and allow further dissemination of tumor cells to seed remote sites.

In addition to the dangers posed by loss of effective treatment time, a cancer patient can be subjected to serious side effects caused by many of the chemotherapeutic agents currently in use. Among these side effects are nausea and vomiting, hair loss, anemia, infection, diarrhea, and blood clotting disorders. Permanent damage to the heart, lung, kidney, reproductive, or other tissue may result.

Tumor cells can sometimes be detected by the presence of "markers"--detectable molecules characteristic of the abnormal metabolic processes in those cells. Many of the markers discovered to date have aided in the early detection of the disease. In some cases, trial and error, combined with epidemiological studies, has enabled physicians to associate a more effective treatment with a particular tumor type. In most cases, however, prediction of efficacy of a particular treatment regimen is difficult, since some tumor cell types may respond to treatment while others do not. Tumor marker levels can be elevated in individuals with benign conditions, and are not specific to a particular type of cancer. The level of a tumor marker can be elevated in more than one type of cancer. Also, tumor marker levels are not elevated in every person with cancer and may be totally undetectable in the early stages of the disease. Tumor markers alone provide no prediction of success for a given treatment regimen.

What is needed in cancer therapeutics is a method for quickly and accurately predicting the effectiveness of a particular chemotherapeutic agent against a patient's individual tumor type.

Summary of the Invention

The present invention describes the discovery that topoisomerase I (TOP1) is multiubiquitinated and destroyed by 26S-proteasome in normal cells. Many tumor cells are defective in this process in response to camptothecin treatment in tumor cells, but that in those tumor cells in which the multiubiquitinated TOP1 is not destroyed by proteolysis, there is an associated and readily detectable accumulation of ubiquitin cross-reactive protein.

The correlation between ubiquitin cross-reactive protein levels and defective destruction of ubiquitin-tagged TOP1 provides a prognostic marker for identification of cancers in which camptothecin and other antineoplastic agents known to act through a DNA-damage mechanism are more likely to provide effective treatment. Since malignant tumor cells exhibit more of the defects leading to uncontrolled cell division than do benign tumor cells, particularly those defects associated with proteolytic regulation in the cell, the present invention also provides a method for distinguishing between malignant tumor cells and benign tumor cells, using ubiquitin cross-reactive protein as a marker for the malignant state.

Camptothecin and other agents associated with DNA damage have previously been considered cytotoxic agents. The defective proteolytic degradation pathway described in the invention, however, is common to cancer cells, rather than all cells, and therefore indicates that these agents target tumor cells more specifically than rapidly proliferating cells. Cellular ubiquitin cross-reactive protein therefore provides a marker for cells for which such anti-tumor agents have therapeutic value.

The invention therefore describes a method for identifying cells sensitive to DNA-damaging agents by assaying to determine the relative level of cellular ubiquitin cross-reactive protein. An elevated level of cellular ubiquitin cross-reactive protein in some cancer cells relative to that found in normal cells is correlated with sensitivity to a DNA-damaging agent. DNA-damaging agents include alkylating agents (e.g., BCNU, CCNU, chlorambucil, cis-platinum,

melphalan, mitomycin C, cyclophosphamide, and semustine), antimetabolites (e.g., thioguanine, thiopurine, hydroxyurea, guanazole, cyclocytidine, ara-C, and 5-aza-2'-deoxycytidine), and topoisomerase II inhibitors (e.g., doxorubicin, daunorubicin, mitoxantrone, menogaril, ribidazine, and VP-16), and topoisomerase I inhibitors (e.g., topotecan, irinotecan, 9-aminocamptothecin, 9-nitrocamptothecin, homocamptothecin, and morpholinodoxorubicin).

More specifically, the relative level of cellular ubiquitin cross-reactive protein as determined by the method is a marker for sensitivity to a topoisomerase I inhibitor such as camptothecin.

The correlation between a higher relative level of cellular ubiquitin cross-reactive protein, as described in the invention, is also used to distinguish malignant tumor cells from benign tumor cells, malignancy being associated with an elevated level of ubiquitin cross-reactive protein as compared to the level of ubiquitin cross-reactive protein found in normal cells.

Cells with a defective ubiquitin/proteasome proteolytic pathway are also identified by the method wherein an elevated level of ubiquitin cross-reactive protein are found in those cells as compared to normal cells. Accordingly, the invention provides a method for identifying cells having a defective ubiquitin/proteasome proteolytic processing pathway, comprising determining the presence of a cellular ubiquitin cross-reactive protein in the cells wherein the presence of the ubiquitin cross-reactive protein correlates with a defective ubiquitin/proteasome proteolytic processing pathway.

Brief Description of the Figures

Figure 1A illustrates a western blot analysis of topoisomerase I (top1) trapped in the form of cleavable complexes in the presence of c camptothecin over a period of time.

Figure 1B illustrates a western blot analysis of topoisomerase I-conjugate to SUMO-1 (small ubiquitin modifiers-1) in the presence of camptothecin over a period of time.

Figure 1C illustrates an immunoblot analysis of levels of topoisomerase I (top1) remaining in cells treated with camptothecin over a period of time.

Figure 1D illustrates a western blot analysis of levels of top2α in cells treated with camptothecin over a period of time.

Figure 2A illustrates immunoblots of topoisomerase I (top1) levels in nine established human breast cancer cell lines treated with camptothecin for different periods of time.

Figure 2B illustrates immunoblots of topoisomerase I (top1) levels in nine established human colon cancer cell lines treated with camptothecin for different periods of time.

Figure 2C illustrates a graphical analysis of the topoisomerase I (top1) levels remaining in human cancer cells of Fig. 2A and Fig. 2B over a period of time after camptothecin treatment.

Figure 3A illustrates western blot analysis of topoisomerase I (top1) levels in three cell lines transformed with different oncogenes and with their non transformed counterpart after camptothecin treatment.

Figure 3B illustrates a graphical analysis of the topoisomerase I (top1) level in cell lines from Figure 3A.

Figure 4A illustrates topoisomerase I (top1) trapped in DNA in the form of cleavable complexes in breast cancer and colon cancer lines after camptothecin treatment.

Figure 4B illustrates topoisomerase I (top1) SUMO-1 conjugates formed in breast cancer and colon cancer lines after camptothecin treatment.

Figure 4C illustrates topoisomerase I (top1) levels in breast cancer and colon cancer lines after camptothecin treatment for various times.

Figure 5A illustrates a graphical analysis of the effect of CPT on transcription arrest and restart in BSC cells.

Figure 5B illustrates a graphical analysis of the effect of CPT on transcription arrest and restart in V79 cells.

Figure 5C illustrates a graphical analysis of the effect of CPT on transcription arrest and restart in BSC cells.

Figure 6A illustrates a graphical analysis of transcription recovery in breast cancer cell line.

Figure 6B illustrates a graphical analysis of topoisomerase I (top1) levels remained after camptothecin treatment in breast cancer cell lines after camptothecin treatment.

Figure 6C illustrates a graphical analysis of the CPT hypersensitivity of ZR-75-1 cells defective in topoisomerase I (top1) down-regulation.

Figure 6D illustrates a graphical analysis of transcription restart in V79 cells treated with MG132 (proteasome inhibitor).

Figure 6E illustrates a graphical analysis of a correlation of topoisomerase I (top1) down regulation and transcription restart in a panel of breast cancer cell lines.

Figure 6F illustrates an analysis of topoisomerase 1 degradation in V79 cells treated with CPT in the presence of MG132 (proteasome inhibitor).

Figure 7 illustrates the accumulation of ubiquitin cross-reactive protein in tumor cells sensitive to camptothecin.

Figure 8 illustrates accumulation of ubiquitin cross-reactive protein in transformed cells.

Figure 9A illustrates western blot analysis of ubiquitin cross-reactive protein (UCRP) using anti-ubiquitin and anti ubiquitin cross-reactive protein (UCRP) antibodies.

Figure 9B illustrates the western blot analysis of ubiquitin cross-reactive protein (UCRP) from different cell lines grown in the presence of IFNβ antibodies.

Detailed Description of the Invention

"Ubiquitin cross-reactive protein" includes cellular proteins which are bound by an antibody that binds to ubiquitin. For example, ubiquitin cross-reactive proteins/ISG15 include cellular proteins which show cross-reactivity with ubiquitin antisera. Specifically, the ubiquitin cross-reactive proteins/ISG15 are about 15 to about 18 kilodaltons (kDa), preferably about 15 to about 17 kDa as measured from Western blot analysis. Specific examples of ubiquitin cross-reactive proteins/ISG15 in the art include those described in Bebington et al. (Mol. Hum. Repro., 10, 966 (1999)), Bebington et al. (Biol Repro., 60, 920 (1999)), Potter et al. (J. Biol. Chem., 274, 250561, (1999)),

Johnson et al. (Biol. Reprod., 61, 312 (1999)), Narasimhan et al. (J. Biol. Chem., 271, 324 (1996), and Haas et al. (J. Biol. Chem., 262, 11315 (1987)). Ubiquitin cross-reactive protein further includes diubiquitin, which consists of two ubiquitin units ligated together (e.g., through the formation of an isopeptide bond between lysine 48 and glycine 76).

Preferably, the ubiquitin cross-reactive protein can be ubiquitin cross-reactive protein/ISG15, as disclosed in Haas et al. (J. Biol. Chem., 262, 11315 (1987). As such, the ubiquitin cross-reactive protein used as a biomarker (i.e., prognastic marker) for tumor cell chemosensitivity can be ubiquitin cross-reactive protein/ISG15, as disclosed in Haas et al. (J. Biol. Chem., 262, 11315 (1987).

The ubiquitin cross-reactive protein has been characterized as an interferon inducible UCRP/ISG15 based on the following observations: (1) Topoisomerase 1 (top1) is ubiquinated and destroyed upon campothecin (CPT) treatment via 26S-proteasome pathway in normal cells; (2) this process is defective in many tumor cells; (3) tumor cells defective in topoisomerase 1 (top1) down regulation are hypersensitive to CPT; (4) there is an up-regulation (or elevation) of UCRP/ISG15 protein in many tumor cells and this upregulation was correlated to top1 down-regulation (destruction); and (5) UCRP/ISG15 was not present in many normal cells or tumor cells resistant to campothecin (CPT).

A preferred agent to detect ubiquitin cross-reactive protein in the methods of the invention is a rabbit antiserum containing anti-ubiquitin antibody which was raised against bovine red blood cell ubiquitin conjugated to keyhole limpet hemocyanin (e.g., Sigma, product No. U-5379). Other preferred agents include antibodies to ubiquitin cross-reactive protein/ISG15.

Ubiquitin cross-reactive protein is a marker for cells with abnormal proteolytic processing mechanisms and for cells which have an increased sensitivity to treatment with cytotoxic agents. Normal cells grow and divide in a controlled manner, primarily due to the cellular regulation mechanisms for proteins involved in cellular reproduction. Among these mechanisms is a proteolytic degradation process commonly known as the ubiquitin/proteasome degradation pathway. The ATP/ubiquitin-dependent non-lysosomal proteolytic pathway of the 26S proteasome utilizes the 8.6 kDa, 76

amino acid protein ubiquitin as a covalent signal to target cellular proteins to the 26S proteasome. Ubiquitin binds the target protein through formation of a peptide bond between its C-terminal glycine residue and the epsilon amino group of a lysine residue in the target protein. Tagging of cellular proteins for proteolytic destruction in the proteasome generally involves the addition of multiple ubiquitin units ligated together through the formation of isopeptide bonds between Lys^{48} and Gly^{76} of successive ubiquitins. The target protein, recognized by the proteasome through its ubiquitin signal, is degraded, while the ubiquitin units are regenerated for use in subsequent proteolytic cycles. Hochstrasser, M. (1996) *Annu. Rev. Genet.* 30, 405-439.

In cancer cells, the proteolytic pathway is defective, leading to deregulation of control of many cellular proteins. Deregulation of cell cycle control proteins, particularly proteins involved in DNA replication, can result in uncontrolled cell division leading to the development of a tumor. A tumor can be either composed of non-cancerous cells (benign) or cancerous cells (malignant). A cancerous cell is a potentially malignant neoplastic cell, characterized by more rapid than normal cellular proliferation, partial or complete lack of structural organization and functional coordination with the normal tissue, and continued unregulated growth after initial stimulation by cellular factors.

Since cancerous cells are characterized by relatively uncontrolled cell growth and DNA synthesis, most of the therapies for treating cancer have been targeted at damage to the DNA of the rapidly-dividing cell. Among these DNA-damaging agents are physical agents, such as radiation, and chemotherapeutics. Among the chemotherapeutics are alkylating agents, which bind to the DNA (often at a preferred guanine residue) and inhibit DNA synthesis. Alkylating agents recognized by the National Cancer Institute include: Asaley, AZQ, BCNU, Busulfan, carboxyphthalatoplatinum, CBDCA, CCNU, CHIP, chlorambucil, chlorozotocin, cis-platinum, chlomesone, cyanomorpholinodoxorubicin, cyclodisone, dianhydrogalactitol, fluorodopan, hepsulfam, hycanthone, melphalan, methylCCNU, mitomycin C, mitozolamide, nitrogen mustard, PCNU, piperazine, piperazinedione, pipobroman,

porfiromycin, spirohydantoin mustard, teroxirone, tetraplatin, thio-tepa, triethylenemelamine,uracil nitrogen mustard, and Yoshi-864.

Antimetabolites, such as 5-fluorouracil (which interferes with DNA synthesis by inhibiting thymidylate synthetase) and cytosine arabinosine (ara-C, which inhibits DNA synthesis through the inhibition of DNA polymerase), are also used for cancer therapy. Antimetabolites also listed with the National Cancer Institute include: 3-HP, 2'-deoxy-5-fluorouridine, 5-HP, alpha-TGDR, aphidicolin glycinate, 5-aza-2'deoxycytidine, beta-TGDR, cyclocytidine, guanazole, hydroxyurea, inosine glycodialdehyde, macbecin II, pyrazoloimidazole, thioguanine, and thiopurine.

A third class of compounds used for cancer therapy is the topoisomerase II inhibitors, such as mitoxantrone, which mediates DNA strand breakage by inhibiting the action of topoisomerase II. Other topoisomerase II inhibitors include doxorubicin, amonafide, m-AMSA, anthrapyrazole derivative, pyrazoloacridine, bisantrene HCl, daunorubicin, deoxydoxorubicin, menogaril, N,N-dibenzyl daunomycin, oxanthrazole, ribidazine, VM-26, and VP-16.

Yet another class of compounds used for cancer therapy is the topoisomerase I inhibitors, exemplified by camptothecin, a number of camptothecin analogs, such as topotecan, irinotecan, 9-aminocamptothecin, 9-nitrocamptothecin, and morpholinodoxorubicin. The inventors have described new topoisomerase I poisons in U.S. application number 09/023,147 (filed February 12, 1998) and PCT application number PCT/US99/02966 ("Heterocyclic Topoisomerase Poisons," filed February 2, 1999).

A DNA-damaging agent is any physical or chemical agent which interferes with the synthesis or repair of DNA, and can include any of the listed therapeutic agents. It is to be understood, however, that this list is not meant to be limiting, since methods of synthesizing and identifying new compounds with alkylating, antimetabolite, or topoisomerase I or II-inhibiting properties are known to those of skill in the art and these new compounds are often reported in the literature.

Ubiquitin Cross-Reactive Protein Levels Correlate with Sensitivity to a Topoisomerase I Poison

The inventors have discovered that an elevated level of ubiquitin cross-reactive protein in cancer cells, as compared to the level of ubiquitin cross-reactive protein in normal cells, is correlated with inability to degrade the ubiquitinated complex formed by the topoisomerase I poison camptothecin and topoisomerase I-DNA. In normal and in some cancer cells in which ubiquitin cross-reactive protein is not found at appreciable levels, camptothecin treatment produces a complex between camptothecin (CPT), topoisomerase I (TOP1), and DNA in which the TOP1 is ultimately polyubiquitinated and degraded. In those cancer cells in which higher relative levels of ubiquitin cross-reactive protein are detected, however, topoisomerase I in the polyubiquitinated complex is not degraded. Many breast and colon cancer cells are defective in TOP1 down-regulation in response to CPT treatment as compared to non-transformed cells. These cells can be identified by the correlated increase in ubiquitin cross-reactive protein in those cells as compared to levels detected in normal cells. The relative level of ubiquitin cross-reactive protein is, therefore, used in the present invention as a predictive marker for determining which tumor cell types will likely respond to treatment with agents known to induce DNA damage.

Ubiquitin has also been associated with proteolytic degradation and regulation of a number of cell cycle proteins. The description of the present invention, therefore, is not intended to be limited solely to detection of cells for which DNA damaging agents will be therapeutically effective, but includes other uses for the ubiquitin cross-reactive protein marker, including detection of cells with abnormal ubiquitin-associated proteolytic degradation processes, which can be useful in both clinical practice and laboratory research. Deregulation of the ubiquitin/proteasome pathway in tumor cells has been documented previously. For example, MHC class I-restricted peptide presentation, which is carried out by proteasome-mediated degradation, is reduced in tumors (Restifo, N.P. et al., J. Exp. Med., 177, 265-272 (1993)). Such a reduction may contribute to escape of tumor cells from immune surveillance (Spataro, V. et al., British J. Cancer, 77, 448-455 (1998)). Cyclins D1 , E and B are known to be elevated in tumors due to decreased proteasome-dependent degradation. (Betticher, D.C., Ann. Oncol.,

7, 223-225 (1996); Clureman, B. et al., Genes Dev., 10, 1979-1990 (1996); Deihl, J.A. et al., Genes Dev., 11, 957-972 (1997); Keyomarsi, K. et al., Proc. Natl. Acad. Sci. USA, 90, 1112-1116 (1993)). Increased proteasome-dependent degradation has also been observed in tumor cells. IκBα, the NF-κB inhibitor, is known to be down-regulated in tumor cells due to increased proteasome-dependent degradation (Palombella, V.J. et al., Cell, 78, 773-785 (1994); Spataro, V. et al., *supra*). The CDK inhibitor p27 is also known to be down-regulated in many colon and breast cancers due to increased proteasome-dependent degradation (Catzavelos, C. et al., Nature Med., 3, 227-230 (1997); Loda, M. et al., Nature Med., 3, 231-234 (1997)). Therefore, deregulation of the ubiquitin/proteasome pathway in tumor cells appears to lead to either elevated or reduced degradation depending on the substrates. Studies of mammalian homologues of DOA4 (a yeast de-ubiquinating enzyme), Unph and Tre2, both of which are oncoproteins and are deregulated in many tumor cells, also indicate an abnormality in the ubiquitin/proteasome pathway in tumor cells (Gupta, K. et al., Oncogene, 8, 2307-2310 (1993); Gupta, K. et al., Oncogene, 9, 1729-1731 (1994); Gray, D.A. et al., Oncogene, 10, 2179-2183 (1995)). The inventors have now demonstrated that many cancer cells are defective in down-regulation of TOP1 by a ubiquitin/proteasome pathway. Furthermore, they have demonstrated that deregulation of ubiquitin/proteasome-dependent TOP1 degradation in tumor cells occurs downstream from the step of ubiquitin-TOP1 conjugation, and that this defect can be correlated with an elevated relative level of cellular ubiquitin cross-reactive protein in those tumor cells.

Camptothecin Forms a Complex with Topoisomerase I Which is Degraded by Proteolysis in Normal Cells, But Not in Regulation-Deficient Tumor Cells

Camptothecin is a quinoline-based alkaloid found in the bark of the Chinese camptotheca tree. It is a naturally-occurring DNA topoisomerase I (TOP 1) inhibitor. Related compounds in the family with topoisomerase I-inhibiting activity include 9-aminocamptothecin, CPT-11, also known as irinotecan, DX-8951f, and topotecan. Many tumor cells have been demonstrated to be hypersensitive to CPT (Pantazis, P. et al., Cancer Res., 53, 1577-1582 (1993); Pantazis, P. et al., Int. J. Cancer, 53, 863-871 (1993)), and the

inventors have demonstrated a correlation between resistance to CPT and extent of TOP1 down-regulation among a panel of breast and colorectal cancer cells **(Fig. 6B).**

Camptothecin (CPT) has been demonstrated to stabilize TOP1 cleavable complexes within an actively transcribed region, and to transiently arrest the elongating RNA polymerase complex. The TOP1 cleavable complex is rapidly multi-ubiquitinated by an E2 or E2/E3 enzyme. Multi-ubiquitinated TOP1 is either released from the DNA template, and destined for degradation by 26S proteasome, or degraded directly on the DNA template by 26S proteasome. Release of multi-ubiquitinated TOP1 from the DNA template may explain the observed redistribution of TOP1 (Danks, M.K. et al., Cancer Res., 56, 1664-1673 (1996); Buckwalter, C.A. et al., Cancer Res., 56, 1674-1681 (1996)), while the majority of ubiquitinated TOP1 molecules as observed in some studies (e.g. in HT29 and ZR-75-1 cells) remained covalently linked to DNA and were found to be released only upon nuclease treatment. In any case, degradation of TOP1 can prevent reformation of TOP1 cleavable complexes in the presence of CPT. Down-regulation of TOP1 therefore confers a tolerant state to cells to the action of CPT.

By forming a complex with topoisomerase I covalently linked to DNA, camptothecin inhibits the rejoining step of the two-step breakage/rejoining process for relaxing superhelical DNA that is normally mediated by topoisomerase I. The essential functions of topoisomerases in DNA replication, RNA transcription, chromosome condensation and segregation depend on their action in breaking/rejoining DNA strands (Wang, J.C., Ann. Rev. Biochem., 65, 635-692 (1996)). Alteration of this breakage/religation reaction by xenobiotics (e.g. anticancer drugs and antibiotics) (Chen, A.Y. et al., Ann. Rev. Pharmacol. Toxicol., 34, 191-218 (1994)), peptide toxins (e.g. CcdB and microcin B17) (Bernard, P. et al., J. Mol. Biol., 226, 735-745 (1992); Vizan, J.L. et al., EMBO J., 10, 467-476 (1991)) and thiol-reactive agents (Frydman, B. et al., Cancer Res., 57, 620-627 (1997)) often results in a unique form of DNA damage, topoisomerase-linked strand breaks. Despite the importance of topoisomerase-mediated DNA damage, little is known about its repair. Camptothecin (CPT) is a specific inhibitor (poison) of eukaryotic topoisomerase I (TOP1) and a potent

anticancer drug (Hsiang, Y.-H. et al., J. Biol. Chem., 260, 14873-14878 (1985)). Its antitumor activity has also been reflected in its preferential cytotoxicity against tumor cells *in vitro* (Pantazis, P. et al., Cancer Res., 54, 771-776 (1994)). However, the molecular mechanism(s) underlying the antitumor activity of CPT remains unclear. CPT is known to inhibit TOP1 by blocking the religation step, resulting in rapid accumulation of the cleavage intermediate, the reversible cleavable complex (Hsiang, Y.-H et al., *supra*; Porter, S.E. et al., Nucl. Acids Res., 17, 8521-8532 (1989)). The formation of ternary TOP1 cleavable complexes in CPT-treated cells results in rapid arrest of both DNA replication and RNA transcription (Hsiang, Y.-H. et al., Cancer Res., 49, 5077-5082 (1989); D'Arpa, P. et al., Cancer Res., 50, 6919-6924 (1990)). Inhibition of DNA synthesis is irreversible and is linked to S-phase-specific cytotoxicity of CPT. The S-phase-specific cytotoxicity has been explained by a model in which collisions between the reversible cleavable complexes and the replication forks trigger irreversible arrest of replication, and formation of double-strand breaks (Hsiang, Y.-H et al., *supra*; Tsao, Y.P. et al., Cancer Res., 52, 1823-1829 (1992); Nitiss, J.L. et al., Mol. Pharmacol., 50, 1095-1102 (1996)).

In many cells, however, the topoisomerase I can be marked for degradation by the addition of multiple units of the ubiquitin protein—a process known as multi-ubiquitination. Camptothecin (CPT)-induced TOP1-mediated DNA damage triggers a ubiquitin-proteasome pathway resulting in multi-ubiquitination and 26S proteasome-dependent destruction (down-regulation) of TOP1. In cells in which topoisomerase I can be degraded by the ATP/ubiquitin-dependent non-lysosomal proteolytic pathway of the 26S proteasome, there is decreased sensitivity to the effects of camptothecin in terms of double-strand damage associated with the covalently-bound complex during DNA replication.

In earlier studies, the inventors had observed that in normal cells treated with anticancer drugs such as camptothecin, TOP1 is multiubiquinated and rapidly destroyed by the 26S proteasome. Desai, S. *et al.*, *J. Biol. Chem.* (1997) 272(39): 24159-24164. Furthermore, down-regulation of TOP1 in response to CPT is reduced in cells transformed by T-antigen, E1A/Raf or Ras. The failure of tumor cells to down-regulate TOP1 correlates with persistent transcription arrest (lack of transcription recovery) and hypersensitivity to CPT.

These results indicate that ubiquitin/proteasome-dependent down-regulation of TOP1 represents a novel repair mechanism for TOP1-mediated DNA damage. Many tumor cells are apparently defective in this "repair" mechanism, and hence hypersensitive to TOP1-mediated DNA damage.

Treatment-Sensitive Cells are Identified by Immunoassay

In the method of the present invention, tumor cells are isolated by methods known to those of skill in the art. Generally, a biopsy is taken from an intact or resected tumor. For Western blot analysis, a single biopsy sample of approximately 2 x 10^5 cells is taken. Primary tumor tissue is stored in liquid nitrogen at -70 degree C. For extraction, the tumor cells are lysed by methods known to those of skill in the art. One such method of extraction from tumor cells has been described in U.S. Patent Number 5,723,302 (Diamandis, issued March 3, 1998). Cells may be lysed directly with SDS-PAGE sample buffer. Cell lysates are then sonicated and subjected to SDS-PAGE analysis using a 20% acrylamide gel, with diubiquitin as control marker. Following transfer of proteins onto nitrocellulose membranes, detection of the control marker and ubiquitin cross-reactive protein bands can be performed using a suitable anti-ubiquitin antibody (e.g., ubiquitin antisera).

Anti-ubiquitin antibodies are readily available from commercial vendors, such as Berkeley Antibody Company (Richmond, CA), Research Diagnostics, Inc. (Flanders, NJ, and Sigma Chemical Company (St. Louis, MO). Since ubiquitin is a highly conserved protein, antibody against any known form of ubiquitin is cross-reactive between species. An elevated relative level of ubiquitin cross-reactive protein indicates a tumor type that is most likely to benefit from anticancer treatment with a DNA-damaging agent (e.g., camptothecin, irinotecan, topotecan, 9-nitro-camptothecin, and related analogs). Alternatively, western blot analysis can be performed using antibodies specific to UCRP/ISG15.

Background levels of ubiquitin cross-reactive proteins/ISG15 are expected to be found in many normal cells. For the average biopsy sample size, however,ubiquitin cross-reactive protein levels in normal cells are generally undetectable by Western blot analysis. Therefore, the increased amount of ubiquitin cross-reactive protein in certain cancer cells, relative to the background

levels found in normal cells, is often defined as detectable presence of ubiquitin cross-reactive protein in cancer cells from an average biopsy as compared to the undetected lower levels of background ubiquitin cross-reactive protein in normal cells analyzed by Western blot.

Ubiquitin cross-reactive protein is a prognostic indicator insofar as ubiquitin cross-reactive protein levels in cancer cells sensitive to DNA-damaging agents such as camptothecin are elevated in relation to background levels of ubiquitin cross-reactive protein in normal cells.

An Elevated Relative Level of Ubiquitin Cross-Reactive Protein Indicates a Cellular Abnormality in the Ubiquitin/Proteasome Pathway

Topoisomerase I, as well as many other cellular proteins, especially those involved in DNA damage and repair mechanisms, is targeted for proteolysis by addition of ubiquitin units in a "branched" fashion, most often utilizing either lysine residue 63 or lysine residue 48 in the ubiquitin protein as the attachment point for multiple ubiquitin units. In cells sensitive to camptothecin, TOP1 is ubiquitinated, but not proteolytically degraded. The defective proteolytic degradation of TOP1 in these cells is correlated with an increased relative level of cellular ubiquitin cross-reactive protein. The marker is therefore used to detect a defective ubiquitin-associated proteolytic processing mechanism, such as that detected in cells with defective proteolytic degradation of TOP1.

The most preferred method for detection is immunoassay, especially Western blot analysis, utilizing anti-ubiquitin antibody (either monoclonal or polyclonal). However, any suitable method for detection of the presence of a ubiquitin cross-reactive protein can be used in the methods of the invention. For example, a ubiquitin cross-reactive protein could be detected using an antibody that specifically binds to a ubiquitin cross-reactive protein without significantly binding to ubiquitin. Particularly, cells are lysed directly with 2x SDS-PAGE sample buffer, then sonicated and subjected to 20% acrylamide SDS-PAGE analysis. Following transfer of proteins onto a nitrocellulose membrane, immunoblotting is performed using a suitable anit-ubiquitin antibody (e.g., ubiquitin antisera) and the ECL Western procedure (Pierce) by standard methods known to those of skill in the art.

Example 1. Determination of CPT-Induced TOP1 Degradation in Tumor Cell Lines

A. Materials and Methods

The human fibroblast cell line WI38 and its SV40-transformed variant 2RA, colon cancer cell lines, HT29, KM12, Colo205, SW480 and HCT116, monkey kidney fibroblast cell lines, BSC, and the Chinese hamster lung cell line V79 were obtained from American Type Culture Collection (Rockville, MD). Breast cancer cell lines, ZR-75-1, MCF7, SKBr3, BT20, T47D, MDA-MB-231, MDA-MB-435, MDA-MB-468 and BT474 and Swiss mouse embryo fibroblast NIH/3T3 and K-ras transformed NIH/3T3 cells (KNIH/3T3) were kindly provided by Dr. K.-V. Chin (Cancer Institute of New Jersey, New Jersey). The mouse fibroblast CB17 cell line, and its E1A/Raf-transformed variant CB17/ER1 and CB17/ER4 were kindly provided by Dr. Stuart Lutzker (Cancer Institute of New Jersey, New Jersey). Monkey kidney fibroblast, all breast and colon cancer cells were grown in RPMI medium supplemented with 10% heat-inactivated fetal bovine serum. V79, CB17, CB17/ER1, CB17/ER4, NIH/3T3, and KNIH/3T3 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum. Monkey kidney BSC cells were grown in Eagle's minimum essential medium supplemented with 10% fetal bovine serum. All media were supplemented with L-glutamine (2 mM), penicillin (100 units/ml), and streptomycin (100 μg/ml). All cultures were grown in a 37°C incubator with 5% CO_2.

B. Uridine Incorporation

Cells (5 x 10^5) were grown in 35 x 10 mm tissue culture plates and treated with 25 μM CPT for various times followed by pulse-labeling with 1 μCi/ml of [5,6-^{3}H] uridine (ICN, 47 Ci/mmole). Following uridine labeling, cells were lysed with 10% TCA and labeled RNA was collected on glass fiber filters. Alternatively, cells were lysed with 4 M isothioguanidine, 0.5% sarkosyl, 2 mM Na-citrate, and 0.1 M β-mercaptoethanol. Samples were directly spotted onto DE81 papers. Filters were washed as described (Desai, S.D. et al., J. Biol. Chem., 272, 24159-24164 (1997)).

C. Immunoblotting of TOP1, TOP2α and TOP1-SUMO-1 conjugates

Cells (10^6/sample) were treated with CPT (25 μM, 1% DMSO) for various time at 37°C. Subsequently, cells were either lysed directly with 2 x SDS PAGE sample buffer or 0.2 N NaOH containing 2 mM EDTA (for monitoring trapped covalent TOP1-DNA complexes) or placed in fresh CPT-free tissue culture media for 30 minutes to reverse the cleavable complexes (for monitoring total cellular levels of topoisomerases) prior to lysis. To detect TOP1-Ub conjugates, cells were lysed in 0.2 N NaOH and 2 mM EDTA. Cell lysates were sonicated and then neutralized with 1/10 volume of 2 N HCl. Immediately following neutralization, one tenth volume of a solution containing 10% NP-40, 1 M Tris, pH 7.4, 0.1 M $MgCl_2$, 0.1 M $CaCl_2$, 10 mM dithiothreitol, 1 mM EGTA, and 100 μg/ml each leupeptin, pepstatin and aprotinin was then added. Neutralized cell lysates were incubated with *Staphylococcus aureus* nuclease (10 μg/ml) for 20 minutes on ice. Reactions were determined by the addition of SDS-PAGE sample buffer (final concentrations: 50 mM Tris-HCl pH 6.8, 15% sucrose, 12 mM EDTA, 3%SDS, and 10% β-mercaptoethanol). Immunoblotting analysis of cell lysates was carried out using TOP1 antisera from scleroderma patients and rabbit antisera against TOP2α as described previously using the ECL Western procedure (Pierce)(Nelson et al., *supra*; Hsiang, Y.-H. et al., Cancer Res., 48, 1722-1726 (1988)). The intensity of each band in the autoradigram was quantitated by densometric scanning.

D. Results

1. CPT induces multi-ubiquitination and degradation of TOP1 in normal and certain cancer cells

V79 cells were treated with 25 μM CPT for various times and were then lysed directly with SDS. Under these conditions, TOP1 was found to be covalently trapped on the DNA (due to formation of covalent TOP1-DNA complexes) resulting in disappearance of the 100 kDa TOP1 band in SDS-polyacrylamide gel (**Fig. 1A**) (the doublet labeled pTOP1 and TOP1 represent the phosphorylated and dephosphorylated forms of mouse TOP1, respectively) (D'Arpa, P. et al., Exp. Cell. Res., 217, 125-131 (1995)). When the same gel shown in **Fig. 1A** was overexposed (five times longer), a ladder of multi-ubiquitinated TOP1 species was observed which is similar to the one described

previously in FM3A (mouse mammary carcinoma) cells (Nelson, W.G. et al., *supra*) **(Fig. 1B)**. In order to measure the total cellular TOP1 levels, CPT-treated V79 cells were replenished with CPT-free media for 30 minutes prior to SDS lysis. Under this condition (CPT removal), TOP1 molecules which were trapped as covalent TOP1-DNA complexes were expected to be released from the complex (reversal)(Hsiang, Y.-H. et al., *supra*), resulting in reappearance of the free TOP1 bands in SDS gel **(Fig. 1C)**. The total TOP1 levels were found to be much reduced (70% reduction in 2 hours) during continuous treatment with CPT, as was observed previously in mouse mammary FM3A cells (Nelson, W.G. et al., *supra*). These results suggest that TOP1 is multi-ubiquitinated and destroyed upon CPT treatment in both normal as well as in a few select cancer cells. We also noticed that while TOP1 was degraded, the TOP2α level was nearly doubled in four hours under the same conditions of CPT treatment **(Fig. 1D)**.

2. CPT-induced TOP1 degradation is deficient in many tumor cells

To investigate whether TOP1 is also multi-ubiquitinated and destroyed in response to CPT treatment in human tumor cells, nine established breast cancer and five colon cancer cell lines were analyzed for their TOP1 levels upon treatment with CPT by immunoblotting **(Fig. 2A)**. These human tumor cells exhibited highly variable extent of TOP1 down-regulation in response to CPT treatment **(Fig. 2A)**. Surprisingly, many breast cancer cell lines such as ZR-75-1 and MCF7 were completely defective in TOP1 down-regulation. Other tumor cells such as 3, BT20, T47D, MDA-MB-468, MDA-MB-435, and MDA-MB-231 exhibited a modest level of degradation (less than 30%) of TOP1 after 6 hours of continuous CPT treatment **(Fig. 2A and 2C)**. The BT474 cells, like FM3A mouse mammary carcinoma cells described previously (Maniatis, T. et al., In Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., p.473 (1982)), exhibited the highest level of degradation (60%) of TOP1 **(Fig. 2A and 2C)**. Similarly, most colon cancer cell lines exhibited reduced extent of TOP1 down-regulation **(Fig. 2B and 2C)**. Colo205 and HCT116 cells were found to be most deficient in degradation of TOP1 (20% degraded in 6 hours) as compared to KM12 cells which showed modest degradation of TOP1 (40% in 6 hours) **(Fig. 2B)**. In contrast to these results, CPT-induced TOP1 down-regulation was

extensive in all non-transformed mouse and human fibroblasts (**Fig. 3**). Within two hours, more than 80% of the TOP1 was destroyed in all these cells (**Fig. 3**).

3. Oncogenic transformation reduces the extent of TOP1 deregulation in response to CPT

Since the extent of TOP1 degradation was found to be highly variable in tumor cells, studies were conducted to investigate whether this phenomenon was related to oncogenic transformation. For this purpose, pairs of cell lines transformed with different oncogenes were used. These cell lines were treated with CPT for different times and TOP1 levels were monitored. As shown in **Fig. 3A**, TOP1 was efficiently degraded (>80% degraded in 4 hours) in normal human fibroblast WI38 cells, in immortalized monkey kidney fibroblast CV1 cells, in mouse fibroblast CB17 cells and in immortalized NIH/3T3 cells.

However, their oncogene-transformed counterparts exhibited reduced extent of TOP1 down-regulation. The 2RA cell line, which is a T-antigen-transformed variant of WI38 cells, was completely defective in TOP1 down-regulation in response to CPT treatment (**Fig. 3A and B**). The ER1 and ER4 cell lines, which are variants of the mouse fibroblast CB17 co-transformed with E1A and Raf oncogenes, exhibited reduced extent of down-regulation of TOP1 as compared with their untransformed CB17 cells (**Fig. 3A and B**). The KNIH/3T3 cell line, the K-Ras-transformed NIH/3T3 cells, exhibited only modest reduction in extent of TOP1 down-regulation (**Fig. 3A and B**).

4. Transcription recovery during prolonged CPT treatment

Down-regulation of TOP1 could conceivably be an efficient mechanism to remove TOP1-mediated DNA damage and to confer tolerance of cells to CPT treatment. CPT is known to cause transcription arrest due to blockage of the RNA polymerase elongation complexes by TOP1 cleavable complexes (Zhang, H. et al., Proc. Natl. Acad. Sci. USA, 85, 1060-1064 (1988); Ljungman, M. et al., Carcinogenesis, 17, 31-35 (1996)). Down-regulation of TOP1 in response to CPT is therefore expected to reduce these transcription roadblocks and to allow transcription restart. We have measured the effect of CPT on transcription arrest and restart. As shown in **Fig. 5A**, BSC cells treated with CPT resulted in rapid arrest of transcription. Within minutes, the rate of transcription was reduced to less than 10% of the untreated control as monitored

by pulse-labeling with ^{3}H-uridine (**Fig. 5A**). Transcription arrest was also confirmed by nuclear run-on assays. However, recovery of transcription (transcription restart) was evident within 15 minutes and continued to increase for the next few hours. At the end of four hours of CPT treatment, the rate of transcription recovered to 22% of the untreated control. VM-26, a TOP2 poison, inhibited uridine incorporation as rapidly as CPT. However, no significant recovery of transcription was observed in the next four hours as evidenced by pulse-labeling with ^{3}H-uridine. Also, no transcription recovery was observed when cells were simultaneously treated with both CPT and VM-26.

To test the hypothesis that transcription recovery following CPT treatment could be due to time-dependent inactivation of CPT in the culture media of treated cells, culture media from CPT-treated cells was transferred to fresh untreated BSC cells and transcription inhibition/recovery was monitored by uridine incorporation. In this experiment, the transcription rate was reduced to 14% of the untreated control within 15 minutes of CPT treatment (**Fig. 5C, sample #4**). Four hours later, the transcription rate recovered to 40% of the untreated control (**Fig. 5C, sample #2**). When CPT-containing media from treated cells (4 hours) (**sample #2**) were transferred to untreated cells for 15 minutes, transcription was again reduced to 14% (**Fig. 5C, sample #5**), suggesting that CPT remained active in the media during 4 hours of incubation. We then tested whether transcription in cells following 4 hours of incubation with CPT became resistant to further CPT treatment. As shown in **Fig. 5C (sample #3)**, when an additional aliquot of CPT (a final combined concentration of 100 μM) was added to cells which already had CPT (50 μM) in the medium for four hours, the transcription rate (measured after another 15 minutes of treatment) was reduced only slightly (from 40%) to 30% of that of the untreated control. Considering the increased CPT concentration (from 50 μM to 100 μM), the BSC cells following four hours of treatment with CPT could be considered in a tolerant state which is much resistant to further CPT-mediated transcription inhibition (without tolerance, the rate should have been reduced to less than 14%). These experiments suggested that transcription recovery in cells treated with CPT was not due to inactivation of CPT but due to development of a tolerant state in CPT-treated cells. This transcription recovery phenomenon is

not unique to BSC cells as we observed similar recovery in V79 Chinese hamster lung cells (see Fig. 5B).

5. Lack of transcription recovery in tumor cells defective in TOP1 down-regulation

To test whether there is correlation between TOP1 down-regulation and transcription restart, breast and colon cancer cells were analyzed for transcription restart during prolonged CPT treatment. As expected, the breast cancer cell line BT474, which is proficient in TOP1 down-regulation (Fig. 6B and 2A), exhibited transcription restart (Fig. 6A) during prolonged CPT treatment. However, transcription restart in another breast cancer cell line ZR-75-1, which was deficient in TOP1 down-regulation (Fig. 6B and 2A), was completely lacking (Fig. 6A). The expanded study using a panel of breast cancer cell lines is shown in Fig. 6E. Again, the correlation between transcription restart and TOP1 down-regulation was observed. These correlative results suggest that transcription restart is due to down-regulation of TOP1 in CPT-treated cells. It is also interesting to note that ZR-75-1 cells which are defective in TOP1 down-regulation, are also hypersensitive to CPT (Fig. 6C).

6. Both down-regulation of TOP1 and transcription recovery depend on 26S proteasome activity

To test whether both down-regulation of TOP1 and transcription restart are dependent on 26S proteasome activity, V79 cells were treated with the 26S proteasome inhibitor, MG132 (Fig. 6D & F). In the presence of MG132, no degradation of TOP1 was observed in V79 cells treated with CPT for four hours (Fig. 6F). Under such a condition, transcription restart was completely blocked (Fig. 6D). Similar results were observed in a mammalian breast cancer BT474 cell line, suggesting that 26S proteasome activity is responsible for both TOP1 down-regulation and transcription restart in CPT-treated cells.

7. Identification of Ubiquitin Cross-Reactive Protein in CPT-Sensitive Tumor Cells

The human fibroblast cell line WI38 and its SV40-transformed variant 2RA, as well as colon cancer cell lines HT29, KM12, and HCT116, were obtained from American Type Culture Collection (Rockville, MD). Breast

cancer cell lines ZR-75-1 and BT474 were kindly provided by Dr. K.-V. Chin (Cancer Institute of New Jersey, NJ).

Twenty-four hours prior to assay, exponential cells (5×10^5) were plated and cultured in 35 x 10 mm tissue culture plates at 37°C in a CO_2 incubator. Subsequently, cells were lysed directly with 2x SDS PAGE sample buffer. Cell lysates were sonicated and then subjected to SDS-PAGE (20% acrylamide) analysis. Following transfer of proteins onto nitrocellulose membrane, immunoblotting was carried out with ubiquitin antisera (antisera raised against bovine red blood cell ubiquitin conjugated to keyhole limpet hemocyanin; Sigma Chemical Co. product No. U-5379) using the ECL Western blotting procedure (Pierce).

Cells (10^6/sample) were treated with CPT (25 mM, 1% DMSO) for various times at 37°C. Subsequently, the drug was removed and the cells were placed in fresh CPT-free tissue culture media for 30 minutes to reverse the cleavable complexes and enable monitoring of total cellular levels of topoisomerases prior to lysis with SDS sample buffer. Immunoblotting analysis of cell lysates was carried out using TOP1 antisera from scleroderma patients, using the ECL Western blot procedure (Pierce). The intensity of each band in the autoradiogram was quantitated by densitometric scanning.

Accumulation of ubiquitin cross-reactive protein was noted in cells deficient in destruction of multiubiquitinated topoisomerase I upon CPT treatment (ZR-75-1, HT29, and HCT 116), while ubiquitin cross-reactive proteins/ISG15 were not found in normal cells and in tumor cells proficient in destruction of multiubiquitinated topoisomerase I (BT474 and KM12) (Fig. 7 and 8).

All publications, patents and patent documents are incorporated by reference herein, as though individually incorporated by reference. The invention has been described with reference to various specific and preferred embodiments and techniques. However, it should be understood that many variations and modifications may be made while remaining within the spirit and scope of the invention.

What is claimed is:

1. A method for identifying cells sensitive to DNA-damaging agents, comprising determining the relative level of a cellular ubiquitin cross-reactive protein, wherein the presence of an elevated level of the ubiquitin cross-reactive protein is indicative of increased sensitivity.

2. The method of claim 1, wherein the DNA-damaging agent is a chemotherapeutic agent.

3. The method of claim 2, wherein the chemotherapeutic agent is a topoisomerase I inhibitor.

4. The method of claim 3, wherein the topoisomerase I inhibitor is camptothecin or a camptothecin analog.

5. The method of claim 2, wherein the chemotherapeutic agent is an alklyating agent.

6. The method of claim 5, wherein the alkylating agent is BCNU, CCNU, chlorambucil, cis-platinum, melphalan, mitomycin C, cyclophosphamide, or semustine.

7. The method of claim 2, wherein the chemotherapeutic agent is an antimetabolite.

8. The method of claim 7, wherein the antimetabolite is thioguanine, thiopurine, hydroxyurea, guanazole, cyclocytidine, ara-C, or 5-aza-2'-deoxycytidine.

9. The method of claim 2, wherein the chemotherapeutic agent is a topoisomerase II inhibitor.

10. The method of claim 9, wherein the topoisomerase II inhibitor is doxorubicin, daunorubicin, mitoxantrone, menogaril, ribidazine, or VP-16.

11. The method of claim 1, wherein the presence of cellular ubiquitin cross-reactive protein is determined using immunoassay.

12. The method of claim 11, wherein the step of determining utilizes monoclonal antibodies to detect ubiquitin cross-reactive protein.

13. The method of claim 11, wherein the step of determining utilizes polyclonal antibodies to detect ubiquitin cross-reactive protein.

14. A method for identifying cells having a defective ubiquitin/proteasome proteolytic processing pathway, comprising determining the presence of a cellular ubiquitin cross-reactive protein in the cells wherein the presence of the ubiquitin cross-reactive protein correlates with a defective ubiquitin/proteasome proteolytic processing pathway.

15. The method of claim 14, wherein the presence of ubiquitin cross-reactive protein is determined using an immunoassay.

16. The method of claim 15, wherein the immunoassay utilizes monoclonal antibodies to detect ubiquitin cross-reactive protein.

17. The method of claim 15, wherein the immunoassay utilizes polyclonal antibodies to detect the ubiquitin cross-reactive protein.

18. A method of distinguishing benign tumor cells from malignant tumor cells, comprising determining the relative level of cellular ubiquitin cross-reactive protein in a preselected sample of said tumor, wherein an elevated level of cellular ubiquitin cross-reactive protein is indicative of a malignant tumor.

19. The method of claim 18, wherein the presence of ubiquitin cross-reactive protein is determined using an immunoassay.

20. The method of claim 19, wherein the immunoassay utilizes monoclonal antibodies to detect ubiquitin cross-reactive protein.

21. The method of claim 20, wherein the immunoassay utilizes polyclonal antibodies to detect ubiquitin cross-reactive protein.

22. The method of any one of claims 1-21 wherein the ubiquitin cross-reactive protein is diubiquitin.

23. The method of any one of claims 1-21 wherein the ubiquitin cross-reactive protein is a protein which is bound by a polyclonal anti-ubiquitin antibody.

24. The method of any one of claims 1-21 wherein the ubiquitin cross-reactive protein is a protein which is bound by a monoclonal anti-ubiquitin antibody.

25. The method of any one of claims 1-21 wherein the ubiquitin cross-reactive protein is a protein which is bound by ubiquitin antisera.

26. The method of any one of claims 1-21 wherein the ubiquitin cross-reactive protein is a protein which is bond by rabbit whole antiserum raised against bovine red blood cell ubiquitin conjugated to keyhole limpet hemocyanin.

27. The method of any one of claims 1-21 wherein the ubiquitin cross-reactive protein is diubiquitin.

28. The method of any one of claims 1-21 wherein the ubiquitin cross-reactive protein is ubiquitin cross-reactive protein/ISG15.

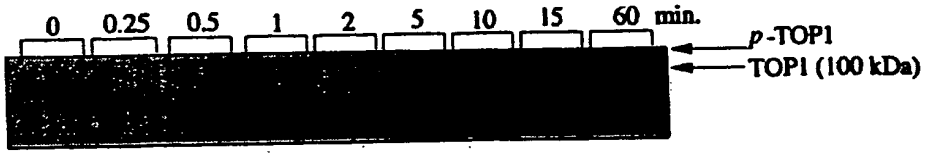

FIG. 1A

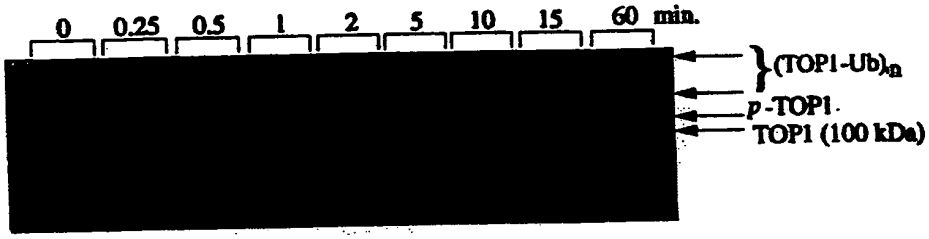

FIG. 1B

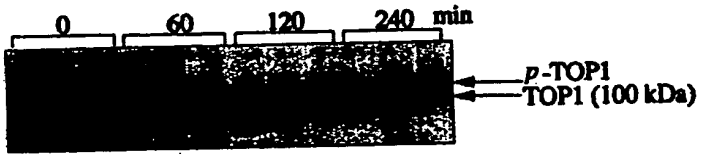

FIG. 1C

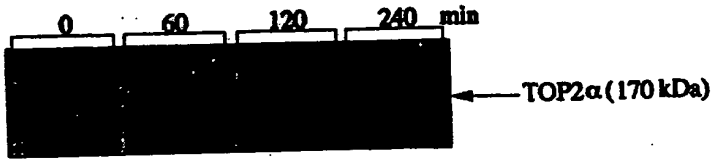

FIG. 1D

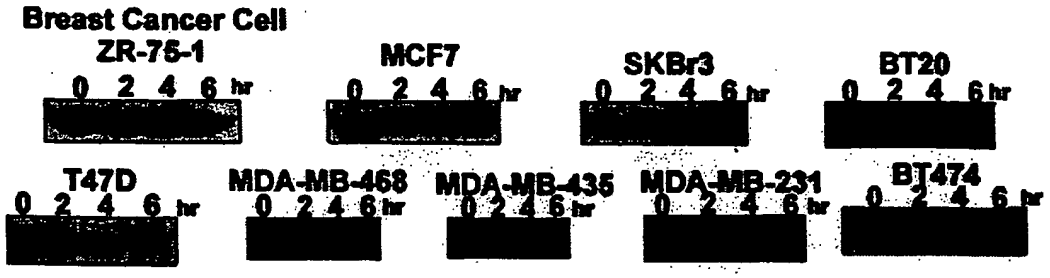

FIG. 2A

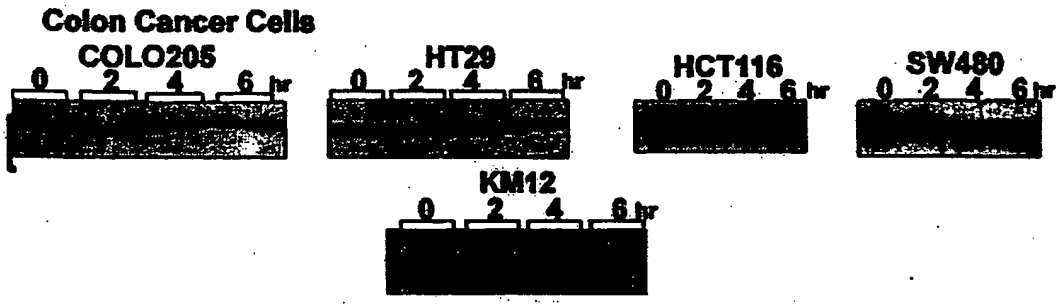

FIG. 2B

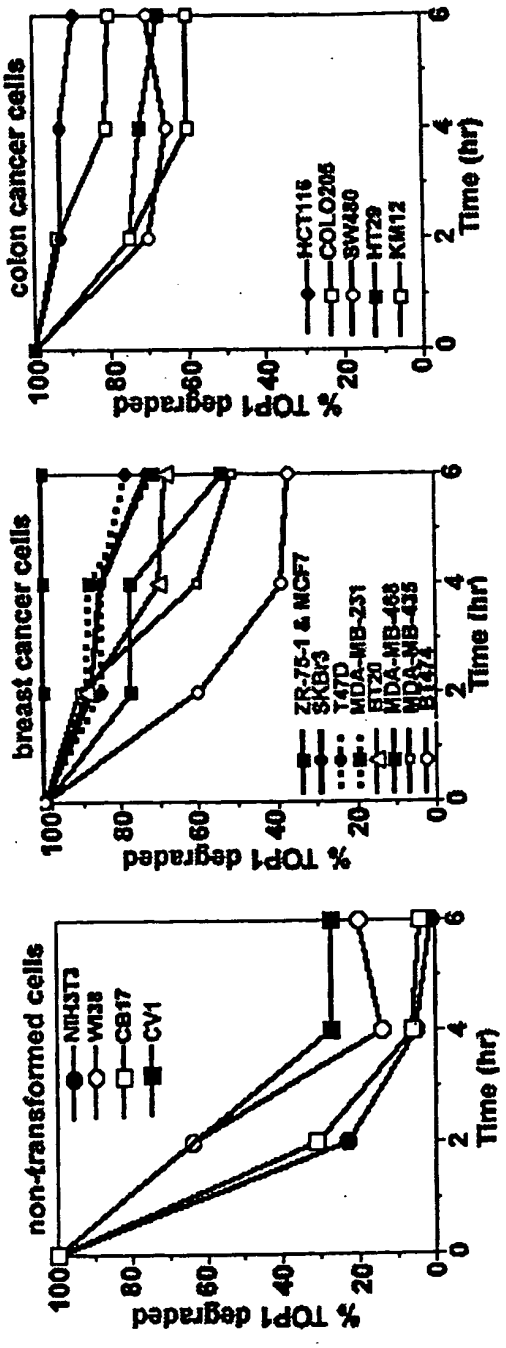
colon cancer cells
% TOP1 degraded
Time (hr)
HCT116
COLO205
SW480
HT29
KM12
breast cancer cells
% TOP1 degraded
Time (hr)
ZR-75-1 & MCF7
SKBr3
T47D
MDA-MB-231
BT20
MDA-MB-468
MDA-MB-435
BT474
non-transformed cells
% TOP1 degraded
Time (hr)
NIH3T3
WI38
CB17
CV1

FIG. 2C

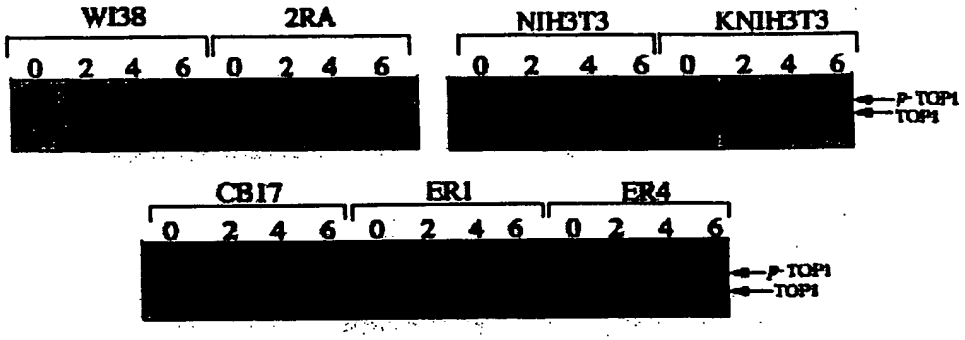
WI38
2RA
NIH3T3
KNIH3T3
0 2 4 6 0 2 4 6 0 2 4 6 0 2 4 6
P-TOP1
TOP1
CB17
ER1
ER4
0 2 4 6 0 2 4 6 0 2 4 6
P-TOP1
TOP1

FIG. 3A

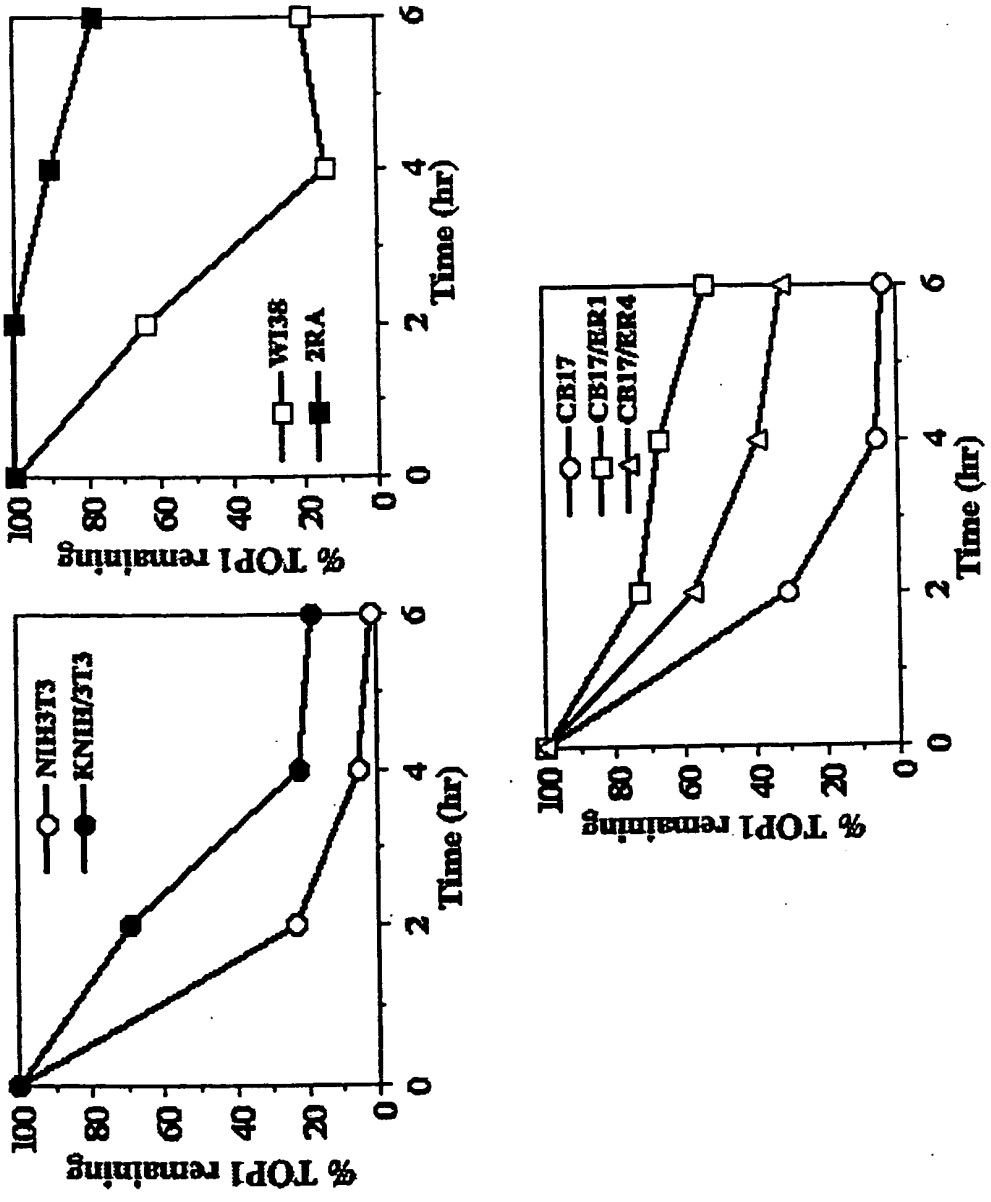
% TOP1 remaining
Time (hr)
WI38
2RA
NIH3T3
KNIH/3T3
CB17
CB17/ER1
CB17/ER4

FIG. 3B

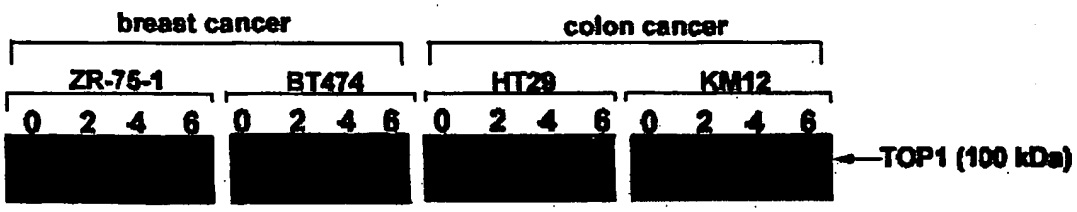

FIG. 4A

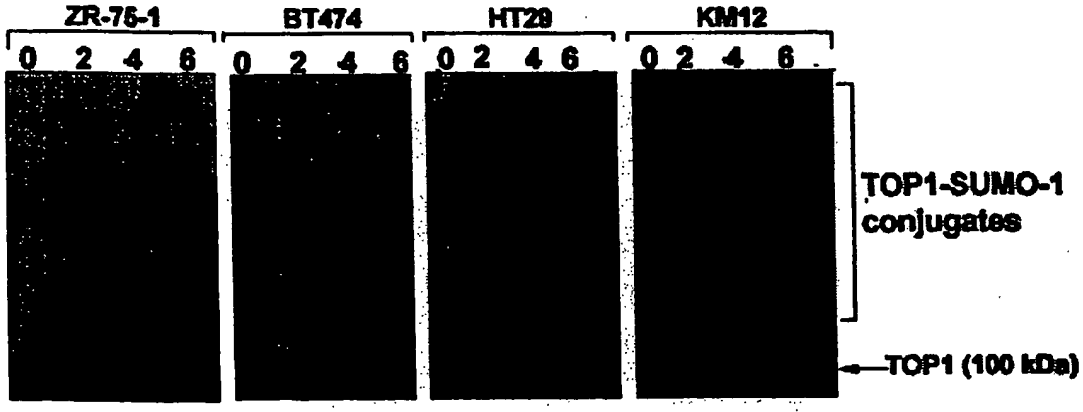

FIG. 4B

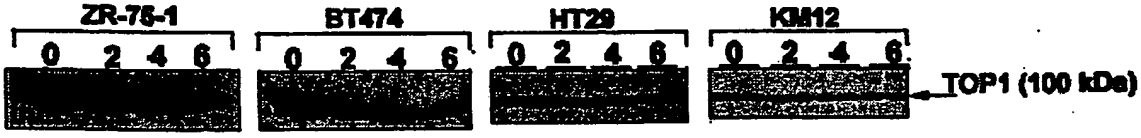

FIG. 4C

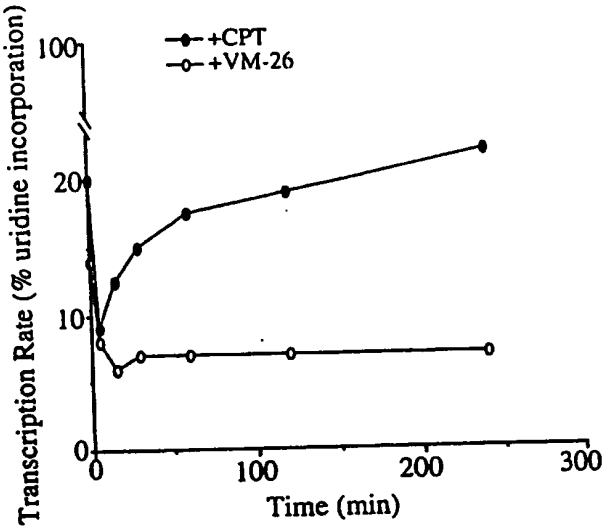
+CPT
+VM-26
Transcription Rate (% uridine incorporation)
100
20
10
0
0
100
200
300
Time (min)

FIG. 5A

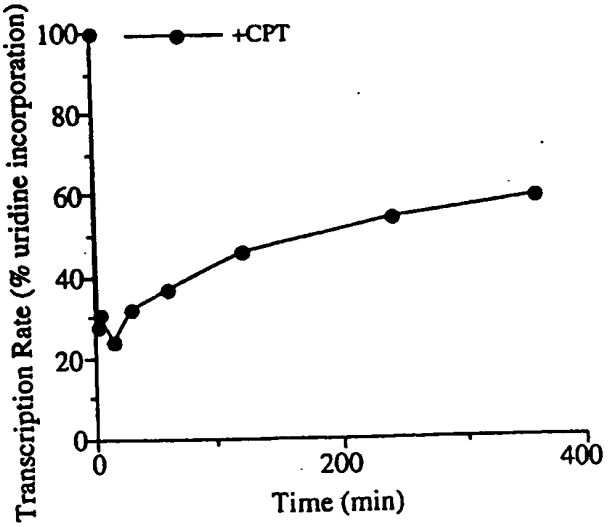
+CPT
Transcription Rate (% uridine incorporation)
100
80
60
40
20
0
0
200
400
Time (min)

FIG. 5B

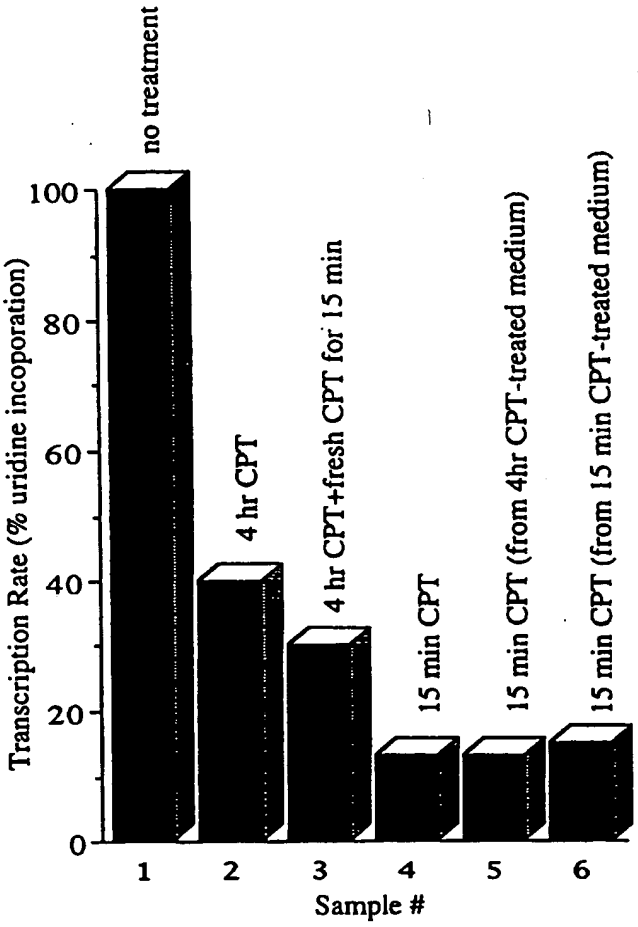
no treatment
4 hr CPT
4 hr CPT+fresh CPT for 15 min
15 min CPT
15 min CPT (from 4hr CPT-treated medium)
15 min CPT (from 15 min CPT-treated medium)
Transcription Rate (% uridine incoporation)
100
80
60
40
20
0
1
2
3
4
5
6
Sample #

FIG. 5C

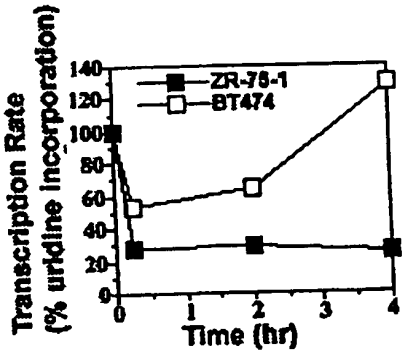

FIG. 6A

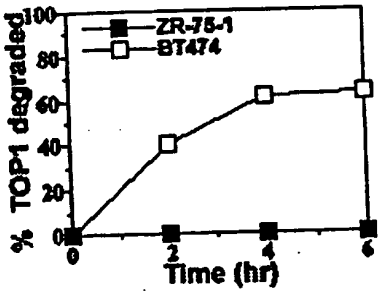

FIG. 6B

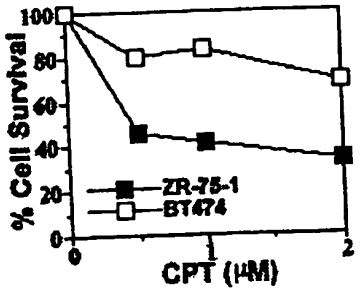

FIG. 6C

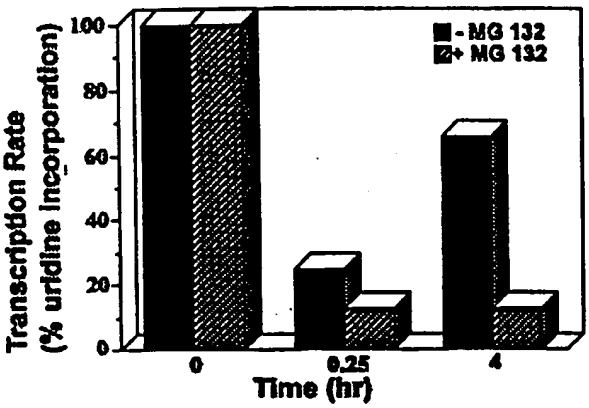
Transcription Rate
(% uridine incorporation)
100
80
60
40
20
0
- MG 132
+ MG 132
0
0.25
4
Time (hr)

FIG. 6D

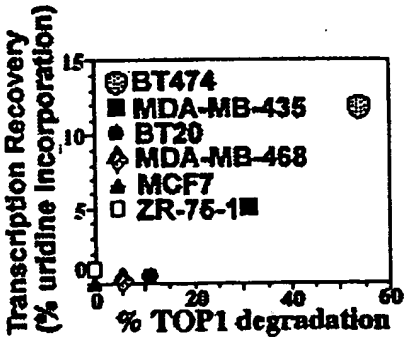
Transcription Recovery
(% uridine incorporation)
15
10
5
0
BT474
MDA-MB-435
BT20
MDA-MB-468
MCF7
ZR-75-1
0
20
40
60
% TOP1 degradation

FIG. 6E

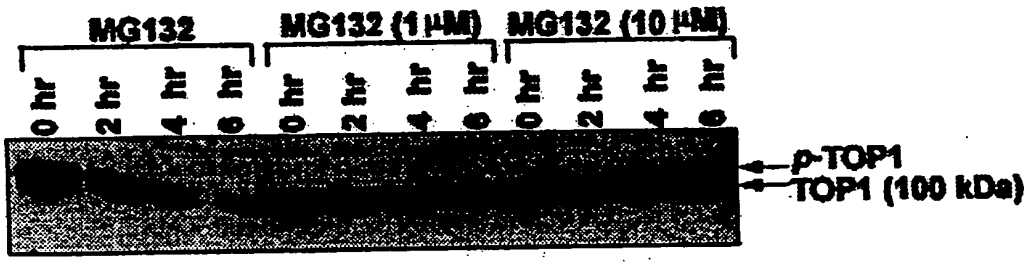
MG132
MG132 (1 μM)
MG132 (10 μM)
0 hr
2 hr
4 hr
6 hr
0 hr
2 hr
4 hr
6 hr
0 hr
2 hr
4 hr
6 hr
p-TOP1
TOP1 (100 kDa)

FIG. 6F

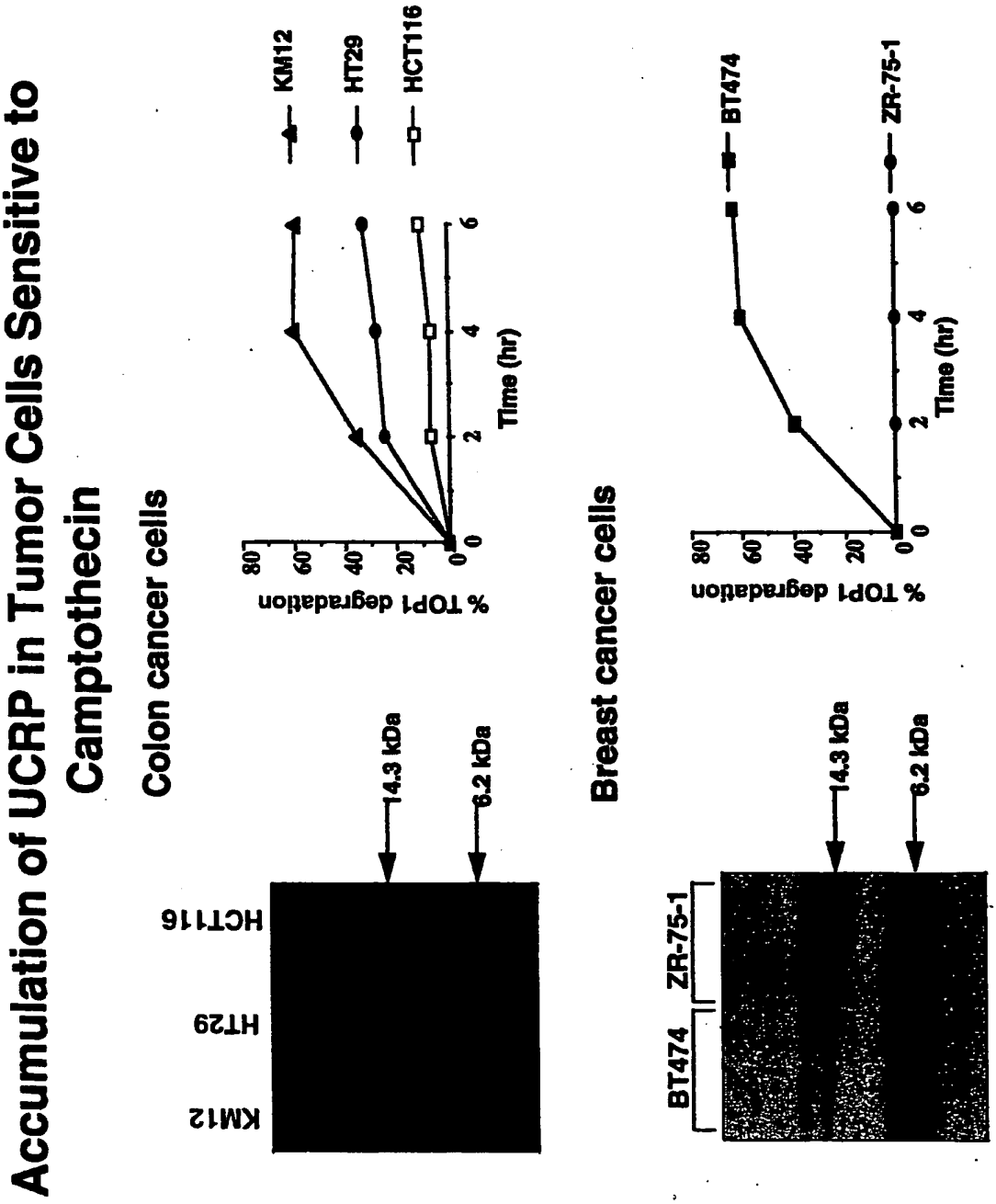
Accumulation of UCRP in Tumor Cells Sensitive to Camptothecin
Colon cancer cells
KM12
HT29
HCT116
% TOP1 degradation
Time (hr)
14.3 kDa
6.2 kDa
Breast cancer cells
BT474
ZR-75-1

FIG. 7

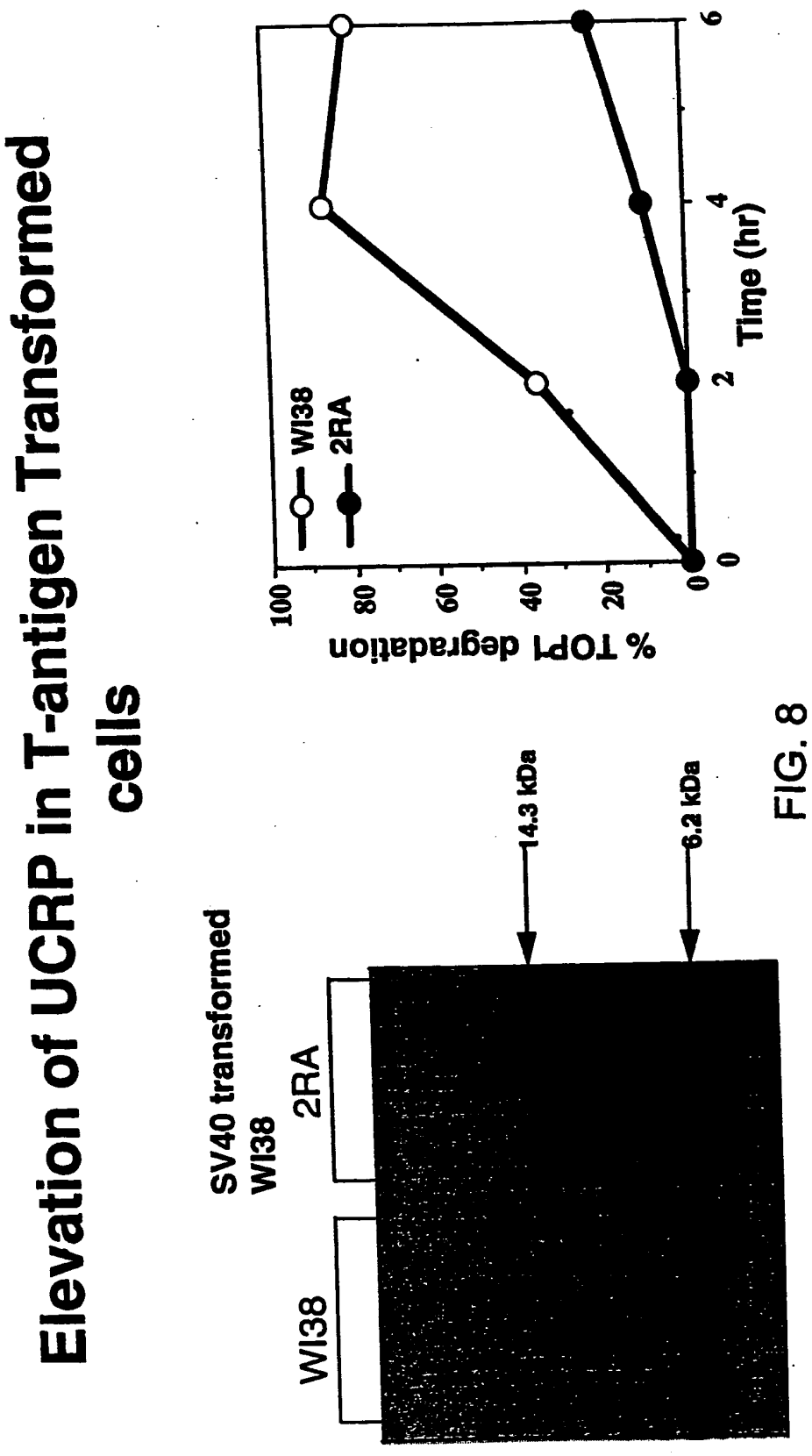
Elevation of UCRP in T-antigen Transformed cells
% TOPI degradation
100
80
60
40
20
0
0
2
4
6
Time (hr)
WI38
2RA
SV40 transformed
WI38
2RA
WI38
14.3 kDa
6.2 kDa

FIG. 8

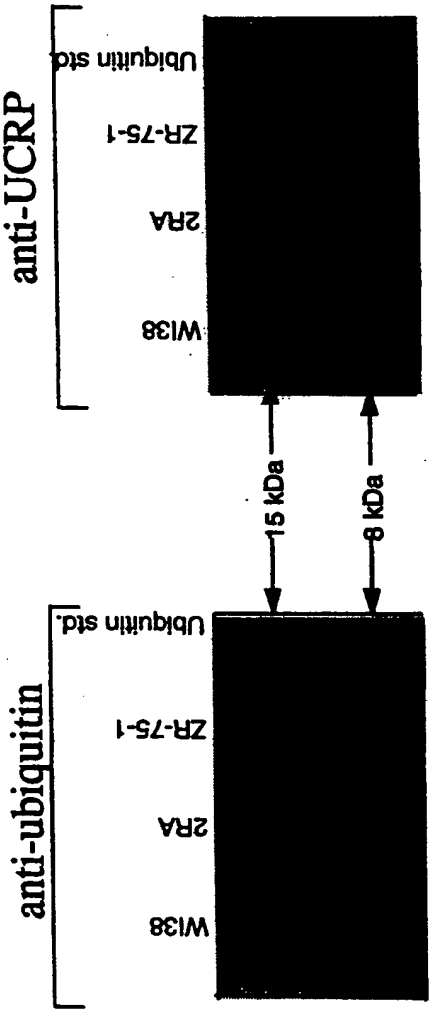
anti-UCRP
Ubiquitin std.
ZR-75-1
2RA
WI38
15 kDa
8 kDa
anti-ubiquitin
Ubiquitin std.
ZR-75-1
2RA
WI38

FIG. 9A

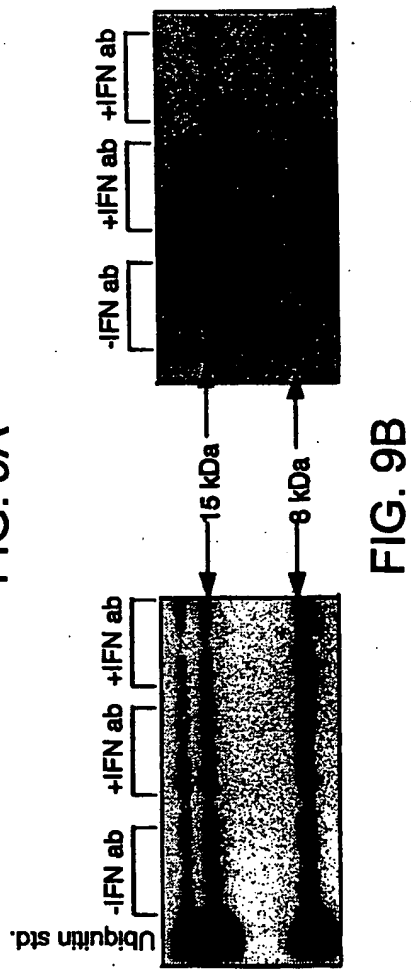
-IFN ab
+IFN ab
+IFN ab
15 kDa
8 kDa
-IFN ab
+IFN ab
+IFN ab
Ubiquitin std.

FIG. 9B

INTERNATIONAL SEARCH REPORT

Internat J Application No
PCT/US 00/09959

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 G01N33/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	DESAI S.D. ET AL.: "Ubiquitin-mediated destruction of topoisomerase I: a potential role of 26S proteasome in the repair of topoisomerase I-mediated DNA damage" PROCEEDINGS OF THE AMERICAN ASSOCIATION FOR CANCER RESEARCH ANNUAL MEETING, vol. 40, March 1999 (1999-03), page 155 XP000912157 abstract -/--	1-28

[X] Further documents are listed in the continuation of box C.

[ ] Patent family members are listed in annex.

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"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
4 September 2000	13/09/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl. Fax: (+31-70) 340-3016	Pellegrini, P

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Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Internat. . Application No
PCT/US 00/09959

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	LIU L.F. ET AL.: "The roles of ubiquitin-dependent proteolysis in determining the sensitivity/resistance of tumor cells to topoisomerase inhibitors" PROCEEDINGS OF THE AMERICAN ASSOCIATION FOR CANCER RESEARCH ANNUAL MEETING, vol. 40, March 1999 (1999-03), page 775 XP000912156 abstract	1-28
P,X	DESAI S.D. ET AL.: "Roles of ubiquitin and ubiquitin-related proteins in camptothecin sensitivity/resistance" PROCEEDINGS OF THE AMERICAN ASSOCIATION FOR CANCER RESEARCH ANNUAL MEETING, March 2000 (2000-03), page 819 XP000912155 abstract	1-28

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